

Amendments to the Specification:

Please replace the paragraph on page 72, line 18 - page 73, line 6 of the specification with the following amended paragraph:

A partial desaturase candidate was isolated using the degenerate primer combination of R0834/R0838, listed in Example 1. The genomic DNA was prepared from Isochrysis galbana CCMP1323 (Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, MA) using the DNeasy DNEASY™ plant maxi kit (Qiagen, Valencia, CA). The I. galbana gDNA was amplified with primers R0834 (5' -GTB TAY GAY GTB ACC GAR TGG GTB AAG CGY CAY CCB GGH GGH- 3') (SEQ ID NO:1) and R0838 (5' -CAT GGT VGG RAA SAG RTG RTG YTC RAT CTG RTA GTT- 3') (SEQ ID NO:10). PCR was carried out in a ~~50~~ \pm 50 μ l volume containing: ~~1~~ \pm 1 μ l of isolated I. galbana gDNA, ~~0.2~~ \pm 0.2 μ M dNTP mix, 50 pM each primer, ~~5~~ \pm 5 μ l of 10 X buffer, ~~1.5~~ \pm 1.5 μ l of 50 mM MgSO₄, and 0.5 U of *Taq* DNA Polymerase. Thermocycler conditions in Perkin Elmer 9600 were as follows: 94 °C for 3 min, then 30 cycles of 95 °C for 45 sec., 55 °C for 30 sec., and 68 °C for 2 min. The PCR amplified mixture was run on a 1.0% agarose gel, and an amplified fragment of approximately 1.1 Kb was gel purified using the Qiaquick QIAQUICK™ Gel Extraction Kit (Qiagen, Valencia, CA). The staggered ends of the fragment were filled-in using T4 DNA Polymerase (LifeTechnologies, Rockville, MD), the isolated fragment was cloned into the pCR-Blunt vector (Invitrogen, Co., Carlsbad, CA), and the recombinant plasmids were transformed into TOP10 supercompetent cells (Invitrogen, Carlsbad, CA).

Please replace the paragraphs on page 73, line 14-page 75, line 19 of the specification with the following amended paragraphs:

To isolate the 5' and 3'-ends, new primers were designed based on the internal sequence of the isolated I. galbana fragment. For the 5 prime end of the gene RO1235 (5'- CGA AGT TGG TGA AGA TGT AGG TGC CG-3') (SEQ ID NO:43) was used, while RO1232 (5'-GAG CGA CGC GTA CAA CAA CTT TCA CGT-3') (SEQ ID NO:44) was used for the 3 prime end of the gene. Approximately ~~1.4~~ 1.4 µg of total RNA was used, according to the manufacturer's direction, with the Rapid amplification of cDNA ends or RACE with the GeneRacer™ GENERACER™ kit (Invitrogen, Carlsbad, CA) and Superscript II™ SUPERSCRIPT™ enzyme (Invitrogen, Carlsbad, CA) for reverse transcription to produce cDNA target. For the initial amplification of the ends, the following thermocycling protocol was used in a Perkin Elmer 9600: initial melt at 94°C for 2 minutes; followed by 5 cycles of 94°C for 30 seconds and 72°C for 3 minutes; 10 cycles of 94°C 30 seconds, 70°C for 30 seconds, and 72°C for 3 minutes; and 20 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 3 minutes; followed by an extension of 72°C for 10 minutes. This first PCR reaction was performed with 10 pMol of RO1235 or RO1232 and GeneRacer™ GENERACER™ 5 prime primer (5'- CGA CTG GAG CAC GAG GAC ACT GA-3') (SEQ ID NO:45), or GeneRacer™ GENERACER™ 3 prime primer (5'- GCT GTC AAC GAT ACG CTA CGT AAC G-3') (SEQ ID NO:46), respectively, with 1 l of Thermozyme™ THERMOZYME™ (Invitrogen, Carlsbad, CA) and ~~1~~ 1 µl of cDNA in a final volume of ~~50~~ 50 µl, according to the manufacturer's directions.

A nested reaction was performed with ~~2~~¹ 2 μ l of the initial reaction, 10 pmol of nested primer RO1234 (5'-AGC TCC AGG TGA TTG TGC ACG CGC AG-3') (SEQ ID NO:47) or RO1233 (5'- GAC TTT GAG AAG CTG CGC CTC GAG CTG-3') (SEQ ID NO:48) and 30 pmol the GeneRacerTM GENERACERTM nested 5 prime primer (5'- GGA CAC TGA CAT GGA CTG AAG GAG TA-3') (SEQ ID NO:49) and GeneRacerTM GENERACERTM nested 3 prime primer (5'- CGC TAC GTA ACG GCA TGA CAG TG -3') (SEQ ID NO:50) respectively, and Platinum TaqTM PLATINUM TAQTM PCRx (Clonetech, Palo Alto, CA) using MgSO₄ according to the manufacturer's protocol. The thermocycling parameter was as follows in a Perkin Elmer 9600: initial melt at 94°C for 2 minutes; followed by 5 cycles of 94°C for 30 seconds and 72°C for 2 minutes; 5 cycles of 94°C 30 seconds, 70°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 68°C for 2 minutes; followed by an extension of 68°C for 10 minutes. Agarose gel analysis of the PCR products showed a band around 800 base pairs for the 5 prime reactions and approximately a 1.2 kilobase band for 3 prime reaction. Subsequent cloning into pCR Blunt (Invitrogen, Carlsbad, CA), transformation into Top10 competent cells (Invitrogen, Carlsbad, CA), and sequencing, revealed an open reading frame with both a start and stop codons.

Primers RO1309 (5'- ATG ATG GAA TTC ATG GTG GCA GGC AAA TCA GGC GC-3') (SEQ ID NO:51) and RO1310 (5'- AAT AAT GTC GAC CTA GTG CGT GTG CTC GTG GTA GG-3') (SEQ ID NO:52) with restrictions sites added for cloning (see underlined EcoRI, and SalI, respectively) were used to isolate a full length gene. As shown above, 10 pmol of primers RO1309 and 1310 were used with Platinum TaqTM PLATINUM TAQTM PCRx

(Clonetech, Palo Alto, CA) using MgSO₄ according to the manufacturer's protocol, with 2 µl of the cDNA as target. The thermocycling parameters were as follows: initial melt at 94°C for 2 minutes; followed by 5 cycles of 94°C for 30 seconds and 72°C for 2 minutes; 5 cycles of 94°C 30 seconds, 70°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 68°C for 2 minutes; followed by an extension of 68°C for 10 minutes. The single product of the reaction was gel purified using the QiaQuick gel purification kit (Qiagen, Valencia, CA), cut with EcoRI and SalI, ligated to pYX242 EcoRI/XhoI linearized DNA with the Rapid ligation kit (Roche, Indianapolis, IN), and designated pRIG-1. The clone pRIG-1 contained a full length gene of 1329 bp (SEQ ID NO:34; Figure 14) and an open reading frame of 442 amino acid (SEQ ID NO:35; Figure 15). (Plasmid pRIG-1 was deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209 on January ____ 14, 2002 under the terms of the Budapest Treaty and was accorded ATCC deposit number ____ PTA-3979.)

Please replace the paragraphs on page 75, line 23-page 76, line 21 of the specification with the following amended paragraphs:

Example 13 Example 14

Expression of *I. galbana* Desaturase Gene in Baker's Yeast

The clone pRIG-1 containing the full-length gene was transformed into the yeast host S. cerevisiae 334 and plated on selective media as described in Example 4. The cultures were grown at 24°C for 48 hours in minimal media

lacking leucine, with 50 ~~■■■~~ 50 μM of exogenous free fatty acid added as a substrate as shown in Table 10. The conversion of substrates was ETA (20:4n-3) to EPA (20:5n-3) and DGLA (20:3n-6) to AA (20:4n-6). The 45.4% conversion to ARA and 59.75% conversion to EPA indicate that this gene encodes for a $\Delta 5$ -desaturase. Table 10 shows some of the fatty acids as a percentage of the lipid extracted from the yeast host. For $\Delta 5$ -desaturase activity, there was little or no background (detection of ARA or EPA observed in the negative control containing the yeast expression plasmid, pYX242.)

Example 14 Example 15

Co-Expression of *I. galbana* Desaturase Gene with Elongases

The plasmid pRIG-1 could be co-transformed with an additional enzyme in the PUFA pathway, such as pRAE-73-A3 which contains the human elongase gene in the yeast expression vector pYES2 as described in Example 4, and co-transformants selected on minimal media lacking leucine and uracil. Substrates such as DGLA or ETA could be added so that the $\Delta 5$ -desaturase would actively produce ARA or EPA, to which the elongase is able to add two carbons to produce ADA or ~~■■■~~-DPA $\omega 3$ -DPA. Therefore, *I. galbana* $\Delta 5$ -desaturase could produce a product in a heterologous expression system that can be used by an additional heterologous enzyme in the PUFA biosynthetic pathway, to produce the expected PUFA.



Table I

Percent amino acid identities shared between $\Delta 6$ -desaturases
and the *S. diclina* $\Delta 6$ desaturase

	<i>S. diclina</i> $\Delta 6$ -desaturase
Phytium- $\Delta 6$	59%
Phaeodactylum tricornatum-$\Delta 6$	42%
Mucor-$\Delta 6$	39%
Rhizopus-$\Delta 6$	39%
Mortierella alpina-$\Delta 6$	39%
Physcomitrella patens-$\Delta 6$	33%
Ceratodon purpurens-$\Delta 6$	36%
Caenorhabditis elegans-$\Delta 6$	31%
Borage-$\Delta 6$	27%
Human-$\Delta 6$	27%



Table II
Accession Numbers of the Listed Sequences:

Sequence	Accession Number
Phytium-Δ6	AAL13310
Phaeodactylum tricornatum-Δ6	AAL92563
Mucor-Δ6	BAB69055
Rhizopus-Δ6	AAP83964
Mortierella alpina-Δ6	AAF08685
Physcomitrella patens-Δ6	CAA11033
Ceratodon purpurens-Δ6	CAB94993
Caenorhabditis elegans-Δ6	AAC15586
Borage-Δ6	AAC49700
Human-Δ6	AAD20018